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Pectin induced transcriptome of a *Rhizoctonia solani* strain causing sheath blight disease in rice reveals insights on key genes and RNAi machinery for development of pathogen derived resistance

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Abstract

Key message **RNAi mediated silencing of pectin degrading enzyme of R. solani gives a high level of resistance against sheath blight disease of rice.**

Abstract Rice sheath blight disease caused by *Rhizoctonia solani* Kuhn (telemorph; *Thanatephorus cucumeris*) is one of the most devastating fungal diseases which cause severe loss to rice grain production. In the absence of resistant cultivars, the disease is currently managed through fungicides which add to environmental pollution. To explore the potential of utilizing RNA interference (RNAi)-mediated resistance against sheath blight disease, we identified genes encoding proteins and enzymes involved in the RNAi pathway in this fungal pathogen. The RNAi target genes were deciphered by RNAseq analysis of a highly virulent strain of the *R. solani* grown in pectin medium. Additionally, pectin metabolism associated genes of *R. solani* were analyzed through transcriptome sequencing of infected rice tissues obtained from six diverse rice cultivars. One of the key candidate gene AG1IA_04727 encoding polygalacturonase (PG), which was observed to be significantly upregulated during infection, was targeted through RNAi to develop disease resistance. Stable expression of PG-RNAi construct in rice showed efficient silencing of AG1IA_04727 and suppression of sheath blight disease. This study highlights important information about the existence of RNAi machinery and key genes of *R. solani* which can be targeted through RNAi to develop pathogen-derived resistance, thus opening an alternative strategy for developing sheath blight-resistant rice cultivars.

Keywords *Oryza sativa* · Polygalacturonase · Transgenic · RNAseq · Silencing

Introduction

Rhizoctonia solani is an important group of soil-borne Basidiomycete fungi infecting a wide range of plant species (Carling et al. 2002; Yi et al. 2002; Zheng et al. 2013; Wang et al. 2015a, b). In rice, it can infect the sheath and leaf tissues at seedling, tillering, and booting stages to cause sheath blight disease, posing a serious threat to rice cultivation worldwide. Hyphae of *R. solani*, a necrotrophic fungus, grow on host surface to form typical lobate appressoria which grow into infection hyphae to cause the disease. The fungus kills the host cells by deploying toxins and enzymes that cause cell death (Marshall and Rush 1980; Vidhyasekaran et al. 1997; Brooks 2007). It is one of the most destructive diseases (Dodds and Rathjen 2010) recording a 5.9–69% yield reduction in rice (Venkat Rao et al. 1990; Tan et al. 2011; Yellareddygari et al. 2014). In the absence

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of effective resistant rice cultivars, farmers rely on chemical fungicides for management of sheath blight disease which causes pesticide run-offs and pollutes the environment.

The approach of development of cultivars possessing resistance against sheath blight disease has not been very encouraging due to lack of high level of resistance in the available rice germplasm, polygenic nature of the trait of tolerance as well as several unknown complexities associated with resistance phenotype. Though, several researchers have attempted to identify the QTLs (quantitative trait locus) (Channamallikarjuna et al. 2010; Silva et al. 2012) and few others developed transgenic rice plants by exploiting the genes of host and pathogen to derive the resistance (Datta et al. 1999; Sridevi et al. 2008; Sripriya et al. 2008; Richa et al. 2017; Tiwari et al. 2017), durable sheath blight resistance is still eluding in rice. Hence, intensive and serious efforts are required to develop effective and durable strategies of resistance against the disease. In view of the limited host plant resistance, alternate approaches of utilizing the pathogen genes by RNA interference (RNAi) and/or genome editing could be an effective strategy to develop resistance against *R. solani*. However, the deployment of RNAi to develop resistance against cellular pathogens like *R. solani* depends on the existence of RNAi machinery in the particular pathogen and the selection of appropriate target genes (Kola et al. 2015; Majumdar et al. 2017).

Since the primary requirement for *R. solani* infection is the degradation of the plant cell wall, it is imperative to target the fungal genes involved in plant cell wall degradation through RNAi to develop good level of resistance against this pathogen. The plant cell wall is composed of cellulose and several matrix polysaccharides including the pectic polysaccharides and the hemicellulosic polysaccharides (Keegstra 2010). Pectins are a key component of the plant cell wall comprising a major co-extensive network in which cellulose microfibrils are embedded (Carpita and Gibeaut 1993; Hadfield and Bennett 1998). Pectinases have been characterized as the most important factor for the pathogenesis of plant fungi which help in decomposition of pectin in the plant cell wall (Herbert et al. 2003; Chen et al. 2017). The degradation of pectin is important not only to weaken the cell wall to facilitate penetration and colonization of the host cell but also is a source of carbon during the pathogen proliferation. Polygalacturonase (PG) is a major pectin degrading enzyme which hydrolyzes the α-1, 4-glycosidic linkage of D-galacturonic acid in pectin. Therefore, the synthesis of PG enzyme by pathogenic fungi is the key for initiation and its establishment during host infection (Chen et al. 2017). The mutation in the PG gene significantly reduced the virulence of *Aspergillus niger* on apple (Liu et al. 2017).The genome-wide transcripts of *R. solani* involved in pectin metabolism have not been studied in detail and such a study would serve as gene resources for targeting the fungus through RNAi or genome editing. Also, the existence of the RNAi pathway in this pathogen has not been reported. Recent studies of the expression profiling of *R. solani* genes and defining their transcriptomes and gene regulatory networks have provided an important lead towards understanding the sheath blight disease and host-pathogen interaction (Xia et al. 2017; Ghosh et al. 2018; Zhang et al. 2018).

In this study, the pectin induced whole genome transcriptome of a highly virulent *R. solani* strain of India was analyzed. The existence of transcripts associated with RNAi machinery has been discovered in this important fungus through this study. The analysis was focused on the identification and characterization of genes encoding enzymes involved in host cell wall degradation and pectin metabolism. Further, an RNAi gene construct for a PG gene was developed and genetically transformed into rice to demonstrate the silencing of pathogen-encoded PG gene and suppression of sheath blight disease development.

Experimental procedures

Sample preparation, sequencing and functional annotation

To catalogue the expressed genes of a highly virulent Indian strain of *R. solani* AG1 IA Wgl-2 (Yugander et al. 2015), fungus was grown in growth medium having pectin only as a carbon source. The sclerotium of fungi was grown in liquid pectin medium (0.7 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 0.001 g $ZnSO_4$, 10 g pectin in 1 L distilled and autoclaved water) as a still culture at 28 °C for 48–72 h and mycelium samples from three biological replicates were pooled to extract the RNA. In order to analyze the expression of *R. solani* genes during host-pathogen interaction, six highly diverse rice genotypes were selected i.e., TN1, BPT 5204, Pankaj, Tetep, N22, and Vandana. Among these, Tetep and Pankaj are tolerant while TN1, BPT 5204, N22, and Vandana are susceptible rice cultivars (Prathi et al. 2018). Freshly grown (4 days old) uniform sized sclerotia of the pathogen were used for inoculation of rice cultivars (at 45 days old stage) by placing them in sheath and leaf tissues. The inoculated plants were maintained in a controlled glasshouse chamber under highly humid and moderate warm conditions to facilitate the disease infection and development. After 5 days of inoculation, the infected sheath and leaf tissues (including 1 cm up and down from the point of inoculation) were harvested from three replicates and pooled for RNA isolation and RNAseq analysis. Total RNA was extracted separately from infected plant tissues and mycelium samples of pure fungal culture using the trizol method. We used three methods for quality assessment of RNA i.e. agarose gel electrophoresis (to test the RNA degradation

and potential contaminations), NanoDrop OD260/OD280 (to test the RNA purity) and analysis using a Bioanalyzer (Agilent Technologies, USA) for RNA quantification and checking the RNA integrity number (RIN). RNA samples without any contamination and RIN value of more than 7.5 were enriched for mRNA by using Oligo (dT) beads and the Ribo-Zero kit to remove rRNA. As required for Illumina sequencing, pair-end (2 × 100 bp) cDNA sequencing libraries were prepared using Illumina TruSeq® RNA 114 Library Preparation Kit as per manufacturer's protocol (Illumina®, San Diego, CA) and sequenced on HiSeq 2500 sequencing system using the commercial facility of Nucleome Informatics Pvt. Ltd., Hyderabad, India. The raw reads of *R. solani* transcripts were filtered to obtain high quality reads (HQR) by removing low-quality reads and adaptors using Trimmomatic v0.32 with default parameters. The HQR from *R. solani* samples were assembled using Trinity v2.0.0 with default parameters (http://trinityrnaseq.sourceforge.net/, Haas et al. 2013). The redundancy of the assembly was reduced through processing by TGICL (TIGR Gene Indices clustering tools) (Pertea et al. 2003). The clean reads were mapped to the reference genomes of *R. solani* AG1 IA available in RSIADB (Chen et al. 2016a, b) and NCBI databases. The fungal transcripts identified from sequencing of pure culture were used to filter out the *R. solani* transcripts from infected rice samples. Gene annotation was performed using BlastP and BlastX with an e-value of less than 1e 50. The Blast2GO software was used to assign the gene ontology terms (GO) to the annotated genes. The KEGG database was used for prediction and identification of various categories of genes. The sequence data have been submitted to NCBI. The BioProject ID is PRJNA523516.

Expression analysis of PG (AGI1A_4727) by qRT-PCR

An aliquot of 1 mg total RNA was reverse transcribed into single-stranded cDNA using the Prime Script RT reagent kit (TaKaRa, Japan). The forward primer 5′CGG GAAAGGGTATCACATTCA3′ and reverse primer 5′GCT TGGTCACACCTCCATTA3′ were designed using online software of Integrated DNA technologies (https://eu.idtdna.com/pages/products/qpcr-and-pcr). The reaction mixture for qRT-PCR was prepared as per the instructions of SYBR premix Ex-Taq kit (TaKaRa, Japan). Reactions were performed in PCR LC-96-well plates (Roche Light Cycler 96; Roche). The 18S *R. solani* ribosomal DNA specific primers were used as internal control (Ghosh et al. 2014).The relative gene expression was analyzed by the ΔΔCt method and fold change was calculated by $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak 2008). Three biological replicates and three technical replicates were used for the experiment. The expression of AGI1A_4727 in *R. solani* infected TN1 sheath tissue was analyzed at a different time interval, i.e. 18 h, 24 h, 48 h, 72 h, 96 h, and 5 days post inoculation and compared with the expression of this gene in *R. solani* grown in PDA (potato dextrose agar) medium.

Gene construct development and genetic transformation of rice

The PG gene (AG1IA_04727) was amplified from the cDNA (obtained from pectin induced *R. solani* Wgl-2 RNA) using gene-specific primers (Supplementary Table S1). The NCBI BLAST was used to check the specificity of AG1IA_04727 in *R. solani* based on sequence similarity. The RNAi vector was developed by cloning the sense and antisense fragment of small interfering RNAs (siRNAs) rich PG gene sequences. In the first step, PCR-amplified PG gene fragment was cloned in pDrive (Qiagen, Netherlands) and pGEM®-T Easy (Promega) cloning vectors using TA cloning strategy. Sense and antisense clones were selected by restriction analysis and sequencing. Sense clones of pDrive and antisense clones of pGEM-T were utilized for the RNAi gene construct development. The pDrive-PG: sense vector was linearized using *Apa*I and *Sac*I restriction enzymes. The pGEM-T clone harbouring PG gene in antisense orientation was also restricted by the same set of restriction enzymes to release gene fragment. The antisense fragment of PG was cloned in the linearized pDrive-PG:sense vector to produce pDrive-PG: sense-antisense. Further, the pDrive-PG: sense-antisense vector was restricted using *Bam*HI and *Sac*I restriction enzymes to release a fragment consisting of sense and antisense fragments of the PG gene and inserted into binary vector pGA3626 (Kim et al. 2009) by T4 ligase. The RNAi binary vector thus produced was mobilized into *Agrobacterium* EHA105 strain. Embryogenic calli of Taipei 309 were transformed using *Agrobacterium tumefaciens* followed by co-cultivation and washing of transformed calli as described earlier (Manimaran et al. 2013). The washed calli were transferred and maintained in selection MS basal salts (Murashige and Skoog 1962) with 2 mg/L 2,4-D, 0.5 mg/L kinetin, 500 mg/L L-proline, 500 mg/L casein hydrolysate, 30 g/L maltose, solidified with 0.3% phytagel and supplemented with 8 mg/L phosphinothricin (Duchefa, Netherlands) for 15 days in dark. After three cycles of selection, resistant calli were transferred to regeneration medium containing MS basal salt, 2 mg/L kinetin, 0.3 mg/L NAA, 30 g/L sucrose, 30 g/L D-sorbitol and 0.4% phytagel. The regenerated plantlets were maintained in the rooting medium (1/2 MS basal salt + 15 g/L sucrose + 0.4% phytagel) and then transferred to hardening medium (Yoshida 1976). The hardened plants were transferred to earthen pots and maintained under controlled conditions in a biosafety glass house.

Genomic DNA extraction, PCR, and Southern hybridization

The molecular characterization of putative PG-RNAi plants was done by PCR. Genomic DNA was isolated using the CTAB method. PCR amplified products were loaded onto a 1.2% agarose gel and checked for the desired fragment of 580 bp corresponding to PG gene (Supplementary Table S1). The Southern blot analysis was carried out as per the standard procedures (Sambrook et al. 1989). Approximately 2–5 µg of genomic DNA was digested with restriction enzymes *Hind* III and *Sac* I, fractionated on 0.8% agarose gel and blotted onto hybond N + nylon membrane (Amersham Pharmacia, Uppsala Sweden) for hybridization. The DIG-labeled PG specific DNA probes were hybridized to confirm the integration of the PG gene construct into the rice genome.

Detection of GFP fluorescence

Fluorescence in seedling roots was observed conveniently with a Leica M205FA stereomicroscope with a Triple beam™ fluorescence illuminator and an FLUOIII™ fluorescence filter system GFP variant. Filter sets used were GFP. Digital pictures were captured with a Leica MC190HD digital video camera and Leica IM software.

Evaluation of RNAi lines for sheath blight disease tolerance

The fresh culture of *R. solani* (strain Wgl-2) was used to inoculate the PG-RNAi lines of rice. For plants inoculation, culturing of the pathogen was done on autoclaved stem pieces (2–3 inches long) of Typha (*Typha angustata*, an aquatic weed) soaked with potato dextrose broth media. These *R. solani* colonized Typha stem pieces were used to inoculate the PG-RNAi plants as per the protocol described in the earlier study (Yugander et al. 2015). The inoculated plants were then kept in a humidity chamber (RH > 95%) at ~ 32 °C for 5–6 days and then shifted to normal glasshouse. After 2 weeks of fungal inoculation, the disease intensity was scored according to the disease rating scale (SES, IRRI 2014). Three replicates with at least three plants of each line in one replicate were evaluated in separate assays. The disease tolerance assay of PG-RNAi plants was also done by detached leaf (Dath 1987) as described in our previous report (Prathi et al. 2018).

Microscopic observations

For analysis of the progression of the disease in PG-RNAi lines and non-transgenic control plants, the leaves were harvested and inoculated with *R. solani* using a detached leaf assay. The leaf samples were placed on filter paper in Petri plates and inoculated with mycelia disc of fresh fungal culture. After infection, leaves were fixed at 24 and 48 h. For fixation, samples were stained with aniline blue 0.1% in 0.1MK_3PO_4 (Hein et al. 2005).The excess dye from the leaves was removed with 0.1M K_3PO_4. Leaves were prepared on to microscopic slides using 10% glycerol. The observation of fungal structures like hyphae and infection cushions were made in the non-transgenic plants as well as in PG-RNAi lines using Leica M205FA microscope and the images were captured using the colored camera (Leica MC190 HD) (Zhang et al. 2018).

RNA extraction and qRT-PCR analysis of PG gene in RNAi lines

Total RNA was extracted from *R. solani* infected plant samples (non-transgenic control plants as well as PG-RNAi lines) after the 96 h of inoculation. The total RNA was isolated from three biological replicates of infected sheath tissue of rice (including the lesion area) using TRIzol reagent (Invitrogen, USA) as described by the manufacturer. The RNA was treated with DNase. The normalized concentration of RNA from each sample was used for cDNA synthesis using ImProm-II™ Reverse Transcription system (Promega USA). The cDNA was normalized for qRT-PCR. The reaction was set using SYBR Premix Ex Taq™ Tli RNaseH plus (TaKaRa, Japan) in a Light Cycler 96 II PCR system (Roche). Real time PCR was performed using AG1IA_04727 gene-specific primers. The relative expression of PG gene was done using gene specific primers (Supplementary Table S1). All experiments were performed with three biological replicates and three technical replicates. The relative expression of the gene was calculated by $2^{-\Delta Ct}$ method, as described in our earlier study (Prathi et al. 2018).

Results

Transcriptome sequencing of *R. solani*

RNA sequencing yielded 38870664 raw reads and 33887530 clean reads. A total of 18488114 (54.56%) reads could be mapped to reference genome while 18271305 (53.92%) reads were unique, suggesting significant molecular divergence of *R. solani* pathotype (AG1-IA) present in India. The sequence statistics are given in Table 1. The clean reads were assembled into contigs and transcript identification. Among the four assembly software used here, the maximum number of transcripts (32,266), as well as large transcripts (≥ 500 bp) was registered by Trinity. The higher number of mean transcript size and N50 transcript length were recorded by EvidentialGene assembler (Table 2). These assembled

Table 1 Summary of raw and high-quality reads of *R. solani* transcriptome data

Particulars	Sequence details
Total raw reads	38870664
Total clean reads	33887530
Total mapped	18488114 (54.56)
Multiple mapped	216809 (0.64)
Uniquely mapped	18271305 (53.92)
Read-1	9086281 (49.73)
Read-2	9185024 (50.27)
Reads map to '+'	9126449 (49.95)
Reads map to '-'	9144856 (50.05)
Non-splice reads	10562231 (57.81)
Splice reads	7709074 (42.19)

transcripts were annotated for biological functions (Supplementary Table S2).

Functional analysis of identified transcripts

The identified transcripts were used for gene ontology to assign biological processes, molecular function and cellular component. Among the cellular component category, 256 terms were categorized into cytoplasm and cytosol, 56 in the endoplasmic reticulum, 12 in Golgi apparatus, 88 in mitochondria, 269 in nucleus and nucleolus, 94 in ribosomes, 1461 in the membrane, 40 in cell and cell wall. Among the molecular function category- heme binding-143, hydrolase activity- 266, iron ion binding – 12, kinase activity-58, ligase activity-19, lyase activity-27, metal ion binding- 116, methyltransferase activity-55, NAD binding-14, nucleic acid binding-96, nucleotide binding 17, oxidoreductase activity- 148, RNA polymerase II transcription factor activity-87, structural constituent of ribosome-74, translation- 39, transporter-64, ATP binding and ATPase activity-457, GTP binding and GTPase activity-90, catalytic activity-89, DNA binding-146, RNA binding-38, zinc ion binding-61, iron-sulfur cluster binding-23, calcium ion binding-21, copper ion binding-21, FAD and FMN binding-82 terms were identified. Among the biological process category, ATP metabolism-27, cell wall modification-12, DNA metabolism-62, ergosterol biosynthetic process-6, fatty acid metabolism-16, lipid and phospholipid metabolism-46, carbohydrate and polysaccharide metabolism-190, metabolic process-92, RNA metabolism-93, protein metabolism-141, transport-170, transcription and translation-146, signal transduction-75, stress-28, chromatin organization and remodeling-22 terms were identified (Supplementary Table S3). Further, these transcripts were categorized into various functions such as enzymes, transcription factors, transporters etc. Among the enzymes, 245 transferases, 42 lyases, 118 synthases, 251 kinases, 42 isomerases, 38 ligases, 75 oxidoreductases, and 214 hydrolases were identified. Among the transcription factors, 5 zinc finger proteins, 8 HLH domain-containing proteins, and 10 bZIP transcription factor domain-containing proteins were identified. Among the carbohydrate-active enzymes (CAZymes), 60 glycoside hydrolases, 28 glycosyltransferases, and 12 polysaccharide lyases were identified. Also, 33 ABC family of transporters, 233 secretary proteins, and 69 cytochrome P450 family proteins were identified (Supplementary Table S4). Interestingly, we identified a Dicer-like protein 1(AG1IA_04057) and two Argonaute-like proteins (AG1IA_04679 and AG1IA_08233). Further, three RNA depended RNA polymerase enzymes were identified (Accession no AG1IA_00255, AG1IA_04803, AG1IA_05207). While performing GO analysis, three transcripts (AG1IA_03474, AG1IA_03475, AG1IA_03476 and AG1IA_03515) were assigned to RISC complex (GO: 0016442) under cellular component category and gene silencing by RNA (GO: 0031047) under biological processes.

Table 2 Summary of de novo assembled transcripts of *R. solani*

Particulars	Trinity	Cd-hit	TGICL	EvidentialGene
Number of transcripts	32266	31298	26009	15821
Total size of transcripts	39487762	38726110	33607459	21391947
Longest transcript	10984	10984	10984	10,984
Shortest transcript	301	301	301	301
Mean transcript size	1224	1237	1292	1352
Number of transcripts > 300 nt	32266	31298	26009	15821
Number of transcripts > 500 nt	23831	23231	19660	12557
Number of transcripts > 1K nt	14644	14425	12697	8145
Number of transcripts > 10K nt	3	3	3	2
N50 transcript length	1756	1775	1846	1881

Identification of genes encoding degradative and pectin metabolism-related enzymes

Taking advantage of an earlier study (Zheng et al. 2013) we searched the genes encoding for degradative enzymes of *R. solani* in our transcriptome database. A total of 125 genes encoding degradation-associated enzymes were identified (Supplementary Table S5). Among the genes involved in pectin metabolism, eight genes (AG1IA_02889, AG1IA_02890, AG1IA_02891, AG1IA_06212, AG1IA_07435, AG1IA_07436, AG1IA_07743, and AG1IA_09779) encoding pectinesterases were identified. Similarly, eight genes (AG1IA_04727, AG1IA_01811, AG1IA_02234, AG1IA_09419, AG1IA_06500, AG1IA_02200, AG1IA_00634, and AG1IA_03368) encoding proteins with polygalacturonase activity were also identified. To analyze the expression of these pectin metabolism–related genes during Rice-*R. solani* interaction, the transcripts of *R. solani* were filtered from the RNAseq data obtained from infected tissues of six rice cultivars and analysis was restricted to fungal genes involved in pectin metabolism. Most of the genes showed a significant level of expression during the infection of six rice cultivars at 5dpi, however, 11 and 5 genes did not show expression in TN1 and Vandana, respectively. This suggests that expression of pectin degradation genes of *R. solani* are highly influenced by rice genotypes (Fig. 1).

Expression analysis of AG1lA_04727

In a previous study, increased expression of AG1IA_04727, AG1IA_01811, AG1IA_02234, and AG1IA_09419 genes after 48 h and 72 h of *R. solani* infection was reported(Zheng et al. 2013). In this study, we analyzed the expression of AG1lA_04727 in infected sheath tissue of TN1 at six time points after fungal inoculation. A sharp increase in the expression level of AG1lA_04727 was observed from 48 h to 96 h after the inculcation. Specifically, the expression was very high during 72 h and 96 h inoculation period. At 5 days after inculcation, its expression reduced as compared to 72 h and 96 h samples, however, it was quite high as compared to the control sample (Fig. 2).

Development of RNAi gene construct and genetic transformation of rice

Identification of RNAi machinery in *R. solani* prompted us to explore resistance development against this deadly pathogen using RNAi approach. The existence of RNAi machinery and the appropriate target gene is the key to success in achieving pathogen-derived resistance using RNAi approach (Kola et al. 2015). The PG-RNAi plant transformation vector was developed which has phosphinothricin (PPT) gene as a plant selectable marker, and green fluorescent protein (GFP) and GUS (β-glucuronidase) as reporter genes. The vector drives the expression of dsRNAs by a constitutive maize ubiquitin promoter. A total of 12,000 embryogenic calli were infected by *Agrobacterium* strain EHA105 carrying the PG-RNAi vector in 18 different batches. Transformed calli were selected on media containing 4 mg/L PPT and a total of 250 putative PG-RNAi plants were regenerated from about the 2500 resistant calli. All the regenerated plantlets were maintained in the transgenic biosafety glass house (Fig. 3).

All 250 putative PG-RNAi rice plants were checked for the presence of the transgene by PCR using *R. solani* PG gene as well as CaMV 35S promoter and bar gene specific primers (Supplementary Table S1). The ~580 bp DNA fragment specific to PG gene was amplified in PG-RNAi plants

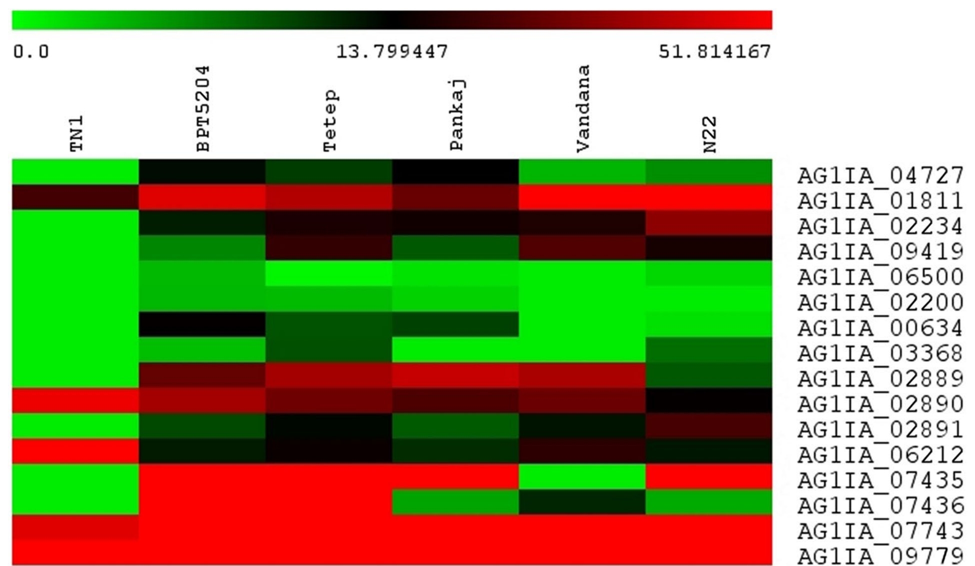

Fig. 1 Heat map showing the expression of *R. solani* genes involved in pectin metabolism. The expression of 16 genes was analyzed in six rice cultivars

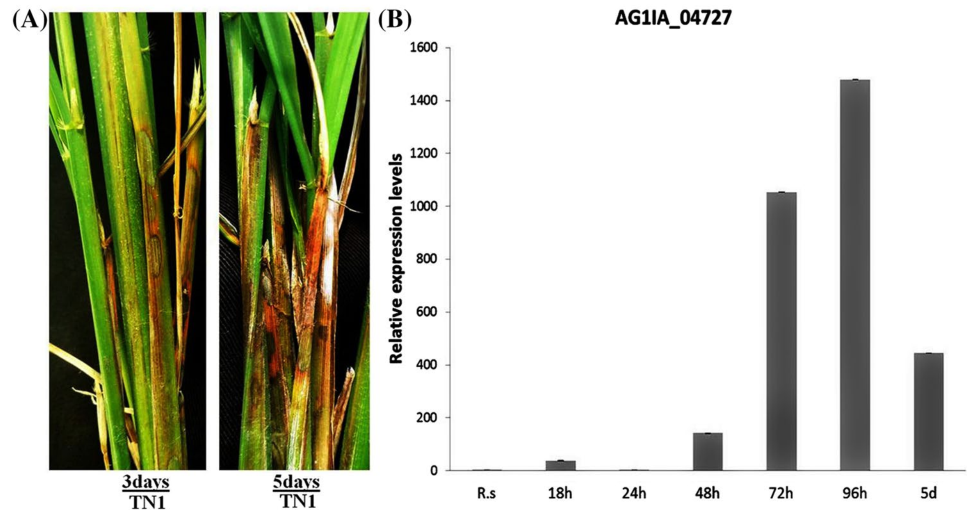

Fig. 2 **a** Symptoms of *R. solani* infection in TN1 rice cultivar at 3 and 5 days post inoculation. Clear visible symptoms were not observed at 18, 24, and 48 h after inoculation. **b** Expression analysis of AG11A_04727 gene at the different time point after *R. solani* infection. X-axis: Samples (R.s.- *R. solani* grown in PDA medium, 18 h to 5d: *R. solani* infected sheath tissue of rice collected at different time point after inoculation) ; Y-axis: Relative expression levels of the gene. Error bars indicate mean ± S.E. of three biological replicates

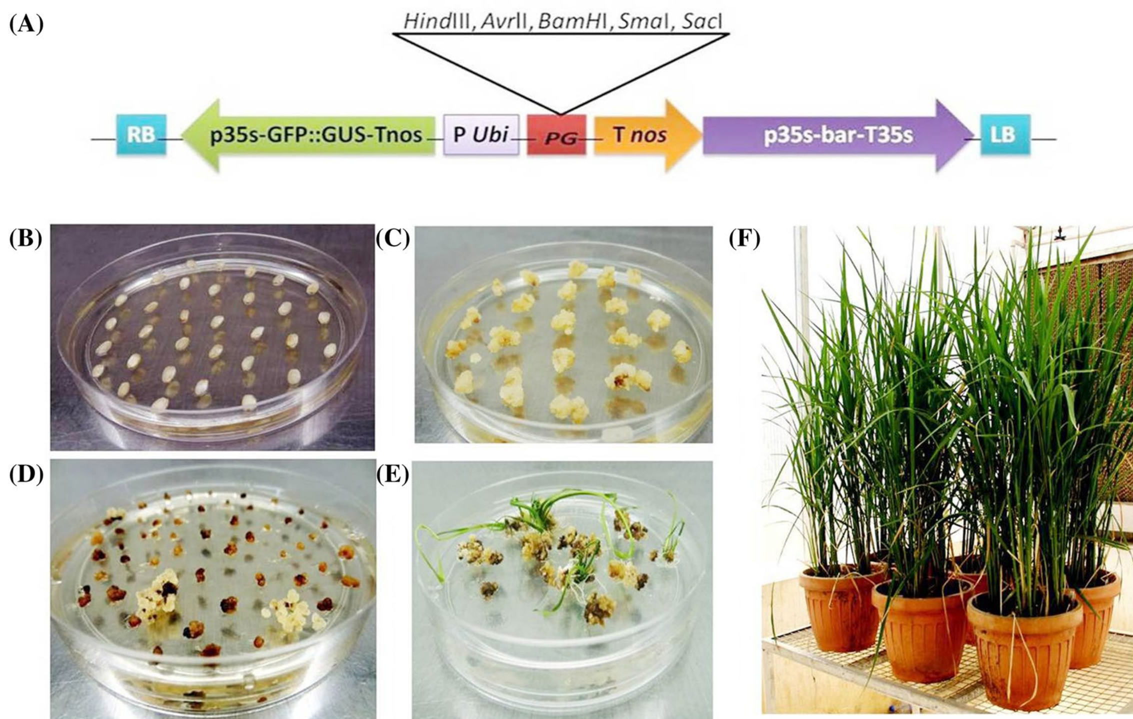

Fig. 3 In vitro regeneration and stable genetic transformation of TP-309 rice plants. **a** Schematic representation of PG-RNAi gene construct; **b** seeds inoculation on callus induction medium; **c** callus subculture; **d** transformed calli growing on selection medium; **e** regenerating callus on selection medium; **f** putative transformants in earthen pots

(Supplementary Fig. S1). For further characterization, 15 PCR positive PG-RNAi plants were selected for analysis by Southern hybridization to confirm the integration of the PG gene into the rice genome. Southern analysis showed the presence of PG gene in four independent T_0 plants namely PG1, PG 2, PG 3, and PG 4. No hybridization signal was detected in the non-transgenic control plant (Supplementary Fig. S1). These four PG-RNAi plants were further tested for the presence of GFP. Seedlings showed fluorescence in transgenic plants under GFP fluorescence (Fig. 4). The non-transgenic plants did not show any fluorescence.

Disease progression in PG-RNAi plants

To evaluate the effects of knocking down the PG gene in the occurrence of sheath blight disease, detached cut leaf assay was performed (Dath 1987). The assay showed a reduced rate of lesion expansion in transgenic plants compared to the

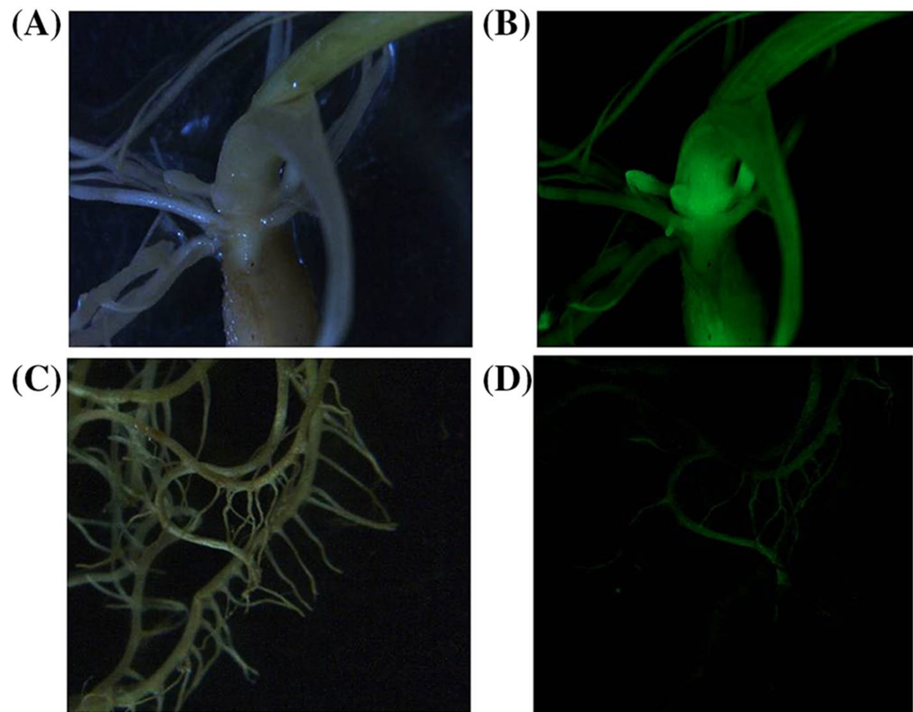

Fig. 4 **a** PG-RNAi plant stem in white light **b** PG-RNAi plant stem under GFP fluorescence **c** PG-RNAi plant roots under white light **d** PG-RNAi plant roots under GFP fluorescence

non-transgenic control plants. The PG-RNAi plants showed a clear difference in disease severity as compared to a non-transgenic control plant at 48, 72 and 96 h after inoculation with *R. solani* Wgl-2 (Fig. 5a). To further confirm the results of detached cut leaf assay, the plants were inoculated by placing the young sclerotium of the *R. solani* Wgl-2 beneath the rice leaf sheath as described in our earlier study (Prathi et al. 2018). The symptoms were recorded at 48, 72 and 96 h after inoculation (Fig. 5b). The progress and severity of disease and disease score were significantly lesser in the PG-RNAi plants (score 3.0) as compared to a non-transgenic control plant (score 9).

Microscopic observations of disease development

The microscopic studies were carried out by visual observations of infected PG-RNAi plants and non-transgenic control plants. After 24 h of inoculation, hyphae grew in bunches

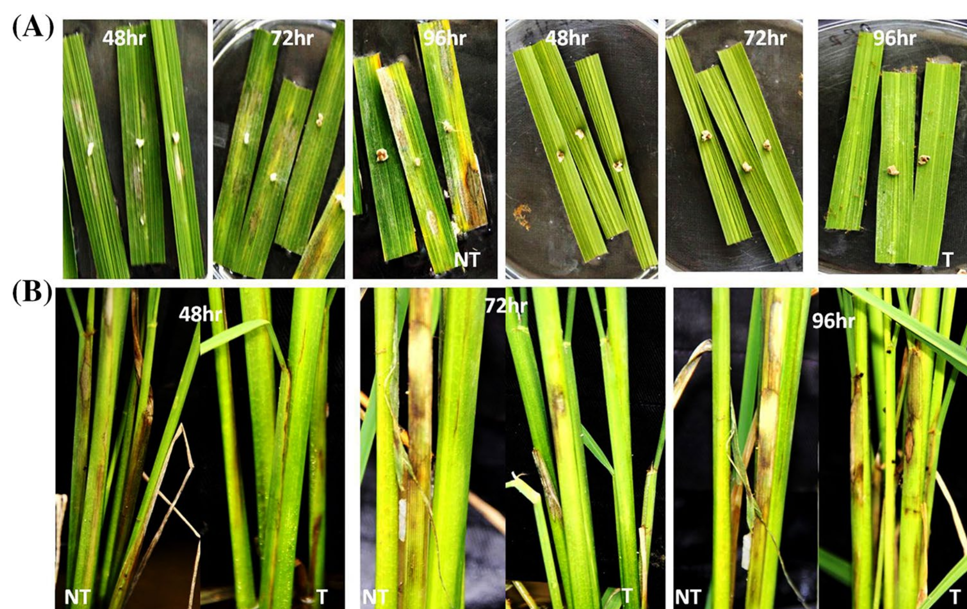

Fig. 5 Evaluation of PG-RNAi lines against the sheath blight disease pathogen *R. solani*. **a** Detached leaf bioassay of PG-RNAi lines and non-transgenic control plant. The *R. solani* sclerotium was placed on the surface of leaf and symptoms were observed at 48, 72 and 96 h after inoculation **b** inoculation of plants by placing the *R. solani* sclerotium or fungal colonized Typha stem pieces in the sheath tissue. Symptoms were recorded at 48, 72 and 96 h after inoculation, T- transgenic RNAi lines, NT- Non-transgenic control

with numerous hyphae on the leaf surface in non- transgenic control plants (Fig. 6a) while they were dispersed and few in number in PG-RNAi plants (Fig. 6b). The hyphae formed side branches and more infection cushions in control plants, whereas PG-RNAi plants showed very few less developed infection cushions. In contrast to PG-RNAi plants, non-transgenic control plants showed extensive colonization of fungal hyphae and appressorium formation (Fig. 6c, d). The cross-section of leaves after 48 h of inoculation showed more fungal hyphae and appressorium in non-transgenic plants (Fig. 6e) as compared to PG-RNAi plants (Fig. 6f). The results clearly demonstrate that the intensity of sheath blight disease was significantly higher in non-transgenic control plants than PG-RNAi plants.

Expression analysis of AG1IA_04727 in fungal-infected PG-RNAi plants

To determine the effect of the host induced silencing on the targeted *R. solani* PG gene expression, the relative expression levels of PG were measured in fungus-infected PG-RNAi and non-transgenic control plants. The mean Ct (cycle threshold) was used for comparing the relative expression of the PG gene in four RNAi lines and non-transgenic control plant. Silencing of PG gene was observed in all the four RNAi lines as compared to the non-transgenic control plant, however, level of silencing varied among the four PG-RNAi lines (Fig. 7).

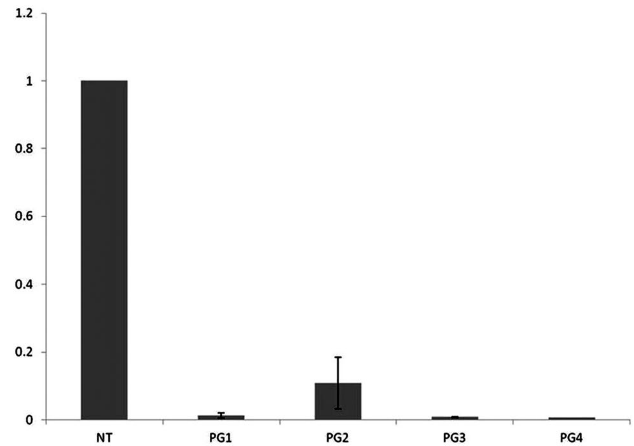

Fig. 7 Relative expression of PG gene in *R. solani* infected non-transgenic plant (NT) and four PG-RNAi lines (PG1 to PG 4) using qRT-PCR. X axis: samples, Y axis: relative expression levels of the gene. Error bars indicate mean ± S.E. of three biological replicates

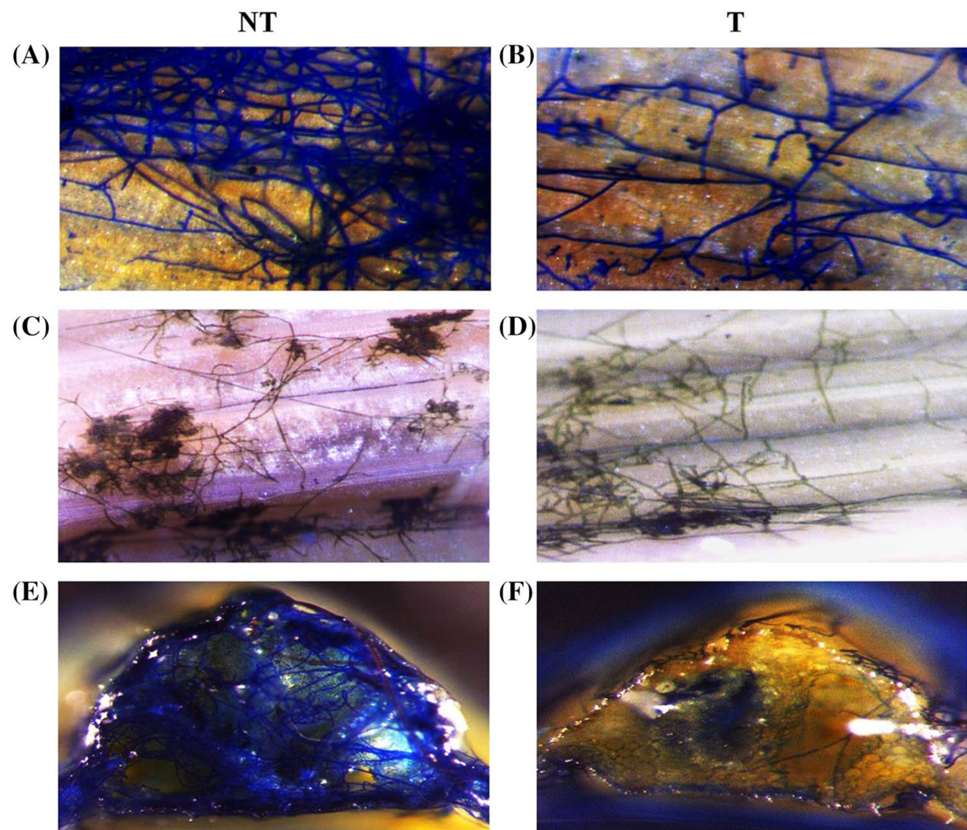

Fig. 6 *Rhizoctonia solani* infection in PG-RNAi and non-transgenic control plants. **a, b** Hyphae developing side branches which directly penetrate on the leaf sheath; **c, d** hyphae initiating infection cushion development at the site of lesion after 24 h of infection **e, f** cross section of leaves showed densely formed infection cushions leading to lesion development and necrosis of the cells in non-transgenic plant after 48 h of infection. T-transgenic RNAi lines, NT-Non-transgenic control

Discussion

The transcriptome analysis of *R. solani AG1* IA provides important evidence to support the molecular basis of host-pathogen interactions and disease establishment. In our previous study, we demonstrated significant variations among the rice infecting strains of *R. solani* present in India based on morphological, pathological, and genetic study among 40 isolates of sheath blight pathogen collected from 12 different states (Yugander et al. 2015). The strains such as Lud-1, Rpr-2, Chn-1, Pnt-1, Rnr-2, and Wgl-2 were categorized as highly virulent strains. Considering the significant variations existing among the rice infecting strains of *R. solani*, it is imperative to analyze the genomics and transcriptomics data of different virulent *R. solani* strains present in India. This is important not only to understand the pathogen biology but also to develop effective strategies for developing durable resistance against the disease.

In this study, we performed RNAseq analysis of a highly virulent strain of *R. solani*, Wgl-2 to identify the repertoire of expressed genes in a pectin-based growth medium. In earlier studies, serious attempts have been made to generate the transcriptome data of *R. solani* AG1 IA from China (Zheng et al. 2013; Zhu et al. 2016; Copley et al. 2017; Liu et al. 2018). In a recent report, transcriptome analysis of an Indian strain BRS1 was performed to identify the pathogenicity determinants of *R. solani* during infection process in rice and the study suggested that functional validation of identified genes would be crucial to get insights about this important pathosystem and to manage this devastating disease (Ghosh et al. 2018). Most of these studies on the transcriptome of *R. solani* AG1 IA were done from infected plant tissues which might have the favorable representation of genes involved in host-pathogen interaction. In this study, a pure culture of pathogen grown in pectin medium was used for RNA sequencing. Therefore, it represents the array of genes involved in growth and metabolism of the pathogen. Further, it is the first such transcriptomic study done in pectin medium as the only carbon source to identify the genes involved in pectin degradation. It should be noted that pectins are a most important constituent of plant cell wall (Carpita and Gibeaut 1993; Hadfield and Bennett 1998) and degradation of pectin is imperative for initiation and establishment of the infection by *R. solani*. Eight genes encoding pectinesterases (AG1IA_02889, AG1IA_02890, AG1IA_02891, AG1IA_06212, AG1IA_07435, AG1IA_07436, AG1IA_07743, and AG1IA_09779) and eight encoding polygalacturonases (AG1IA_04727, AG1IA_01811, AG1IA_02234, AG1IA_09419, AG1IA_06500, AG1IA_02200, AG1IA_00634, and AG1IA_03368) were identified. The up-regulation of the genes AG1IA_04727, AG1IA_01811, AG1IA_02234, and AG1IA_09419 at 48 h and 72 h of infection in rice was reported (Zheng et al. 2013), suggesting an important role of these genes in the pathogenesis of *R. solani*.

The whole genome transcriptome helped us to unravel the RNAi machinery in *R. solani*. Four core RNAi components i.e., RdRP, Dicer, Argonaute and RISC are considered a hallmark of the existence of RNAi pathways in organisms. The conserved RNAi pathway has undergone various adaptations during the fungal evolution and it is significantly diversified among various fungal species because of the numbers of RNAi pathway proteins or enzymes (Nakayashiki and Nguyen 2008). As the reports on the existence of RNAi machinery in fungi suggest that not all the fungi may have the complete RNAi pathway, we were keen to identify the transcripts associated with the RNAi pathway in *R. solani*. Notably, *Ustilago maydis*, the causal organism of corn smut shows complete loss of RNA silencing machinery while budding yeasts do not have RdRP (Dang et al. 2011; Majumdar et al. 2017). Such fungi may not respond to RNAi based approaches of gene silencing. Hence, it is most crucial to understand if the target fungus has the full complement of RNAi machinery before the initiation of work on development of resistance through plant-based transgenic RNAi approaches (Majumdar et al. 2017). In this study, we identified Dicer-like protein 1(AG1IA_04057), Argonaute-like proteins (AG1IA_04679 and AG1IA_08233), RdRPs (AG1IA_00255, AG1IA_04803, AG1IA_05207) and proteins associated with RISC (AG1IA_03474, AG1IA_03475, and AG1IA_03476) which provides ample scope to hypothesize that RNAi based approaches could be successful against *R. solani*. These genes are an important source of information to further probe the mechanism of RNA silencing pathway in *R. solani*. The success in the silencing of pathogenicity MAP kinase 1 (PMK1) homologues RPMK1-1 and RPMK1-2 of *R. solani* through host delivered RNAi could be attributed to the functional RNAi machinery present in *R. solani* (Tiwari et al. 2017).

In the absence of host plant resistance, RNAi based pathogen-derived resistance can be an alternate approach to develop resistance against sheath blight disease in rice. Despite serious efforts made to identify the host plant resistance by the screening of a large number of rice genetic resources, success in terms of developing commercial rice cultivars with desirable sheath blight disease resistance or tolerance could not be achieved. Rice lines with partial resistance to the disease has been reported earlier (Srinivasachary 2011) and some of them have been exploited to map the QTLs associated with partial resistance/tolerance and for identification of genetic markers for introgression of these genetic segments into popular rice cultivars (Sato et al. 2004; Pinson et al. 2005; Liu et al. 2009; Yin et al.

2009; Channamallikarjuna et al. 2010; Wang et al. 2012; Taguchi-Shiobara et al. 2013; Chen et al. 2014). However, sufficient level of resistance could not be realized in these studies due to the complex and polygenic nature of sheath blight resistance. Similarly, extensive efforts have been made to exploit the cis or transgenic derived resistance. Many of the plant-derived resistance genes have been over-expressed to develop sheath blight resistance in rice (Datta et al. 1999; Datta et al. 2001; Sridevi et al. 2008; Sripriya et al. 2008; Shah et al. 2009, 2013; Richa et al. 2017; Xue et al. 2016). However, most of these efforts could not translate into development of commercial sheath blight resistant rice cultivars. Therefore, relying on pathogen-derived resistance using RNAi may prove to be a more successful approach for development of durable sheath blight resistant cultivars. The success of RNAi based resistance against the fungal pathogen is primarily dependent on the existence of RNAi machinery in pathogen and the target gene. In this study, we identified the genes encoding core RNAi pathway proteins and enzymes from the transcriptome data of *R. solani*. Further, we selected one of the genes involved in pectin degradation as a target for RNAi based pathogen-derived resistance. The AG1IA_04727 encoding polygalacturonase was used for the construction of RNAi vector and genetic transformation in rice. It should be noted that expression of AG1IA_04727 was induced after 48 h and 72 h of infection in rice (Zheng et al. 2013). In our study also, AG1IA_04727 showed 1052, 1478, and 444-fold up-regulation at 72 h, 96 h, and 5 days after inoculation, respectively. This suggested that AG1IA_04727 may be a key gene involved in plant cell wall degradation during the commencement and establishment of sheath blight disease by *R. solani*.

Four PG-RNAi lines were developed in the genetic background of the Japonica cultivar, Taipei 309 through genetic transformation. These lines showed a high level of resistance and were better than the non-transgenic control plants as demonstrated by detached leaf assay and whole plant inoculation. Further, microscopic and symptomatology studies suggested that AG1IA_04727 may be one of the key pathogenicity determinants of *R. solani*. Reduced lesion size and delayed symptoms development leading to significantly diminution in disease severity was noticed in PG-RNAi lines. This was further confirmed through observation of very few and dispersed hyphae with fewer branches and infection cushions, and appressorium in PG-RNAi plants. We observed that the site of infection cushion developed only in the non-transgenic control plant while PG-RNAi plants did not show any visible lesions at 24 h of inoculation. Interestingly, in couple of recent reports, rice encoded polygalacturonase-inhibiting proteins (PGIP) inhibiting fungal polygalacturonase activity were over-expressed to enhance rice resistance to the sheath blight disease (Wang et al. 2015a, b; Chen et al. 2016a, b). PGIPs are extracellular leucine-rich repeat proteins that can restrain the degradation of the plant cell wall by counteracting secreted polygalacturonases from pathogens. These studies showed that overexpression of rice PGIP significantly enhanced the resistance against *R. solani* by inhibiting the tissue degradation by fungal polygalacturonases, and the levels of disease resistance matched with the expression levels of PGIP in the transformed rice lines (Wang et al. 2015a, b; Chen et al. 2016a, b). These studies suggest that inhibiting the fungal PG would be crucial in combating the rice sheath blight disease. However, obtaining the complete inhibition of fungal PG by rice encoded PGIP may not be possible due to several reasons such as difference in the expression level of PG and PGIP in fungus and rice, activity and interaction ability of PGIP, expression variation of PGIP in different tissues of rice, and evolution of PGs in fungus to escape the plant PGIPs. Further, the expression level of PGIP in transgenics will be a limiting factor in obtaining high level of resistance. Therefore, direct targeting of fungal PG by RNAi approach seems to be a more effective and viable strategy for inhibition of secreted polygalacturonases by *R. solani*.

Overall, this study brings important information about the RNAi pathway in *R. solani* and possible candidate genes as a target for RNAi mediated pathogen-derived resistance. The identified genes associated with gene regulation, pathogenesis, degradative enzymes, and growth and metabolism of *R. solani* can be important target genes for RNAi based approaches. The pectin induced transcriptome of highly virulent Indian strain of *R. solani* (Wgl-2) will facilitate to understand the pathogen biology and evolving more effective strategies to develop sheath blight disease resistance in the Indian subcontinent. Further, RNAi based silencing of AG1IA_04727 encoding polygalacturonase suggest that it is an important pathogenicity determinant of *R. solani* and can be exploited to develop resistance against sheath blight disease in elite rice cultivars. Our study provides an important advancement in this field and will serve as a much-needed catalyst to develop the necessary tools for sheath blight disease resistance development in rice.

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Author contributions Conceived and designed the experiments: SKM, TBR, and SMB. Performed the experiments: TBR, RC, RM, EP, VV, BS, MRR, AY, GSL, SKM, and DL. Analysis of data: SKM, TBR, RM, RC, MSM, and RMS. Wrote the article: SKM, TBR, SMB, RMS, GSL, and MSM.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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